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REGULAR ARTICLE

SCREENING OF *ADHATODA VASICA* NEES AS A PUTATIVE HIV-PROTEASE INHIBITOR

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SUMMARY

AIDS remain as one of the greatest public health challenges in the current world's health sector. *Adhatoda vasica* Nees, is widely used as a folk medicine and in Ayurvedic system of medicine in India. As a part of our prescreening program of anti-HIV agents from natural sources, various crude extracts of *A. vasica* were evaluated in-vitro. Pepsin Assay as a substitute of HIV-Protease was used for screening HIV-protease inhibitors in this experiment. The crude extract of *A. vasica* exhibited potent inhibitory activity of enzyme Pepsin in this assay system, so it might be a potent inhibitor of HIV-Protease which belongs to same aspartate family of enzyme and sharing same signature group at the active site.

Key words: HIV-Protease, HAART, AIDS, Pepstatine A

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1. Introduction

A large number of research groups is actively involved globally in the investigation of anti-HIV agents that interfere with different stages of HIV replicative cycle (Balzarini et al., 1986; Sarin, 1988). A number of anti-HIV drugs used in conventional AIDS therapy are available in the market, unfortunately the administration of these compounds clinically to the AIDS patients exhibited serious side effects (Scinazi R et al. 1992). As the data show the rapid spread of the AIDS epidemic and the appearance of HIV strains resistance to the currently available drugs suggests that an effective and durable chemotherapy will require the use of innovative combinations of drugs having diverse mechanism of anti-HIV activity (Tantillo et al., 1994; Balzarini et al., 1996; Lipsky, 1996) and a continuous need for alternative inhibitors. New anti-HIV agents with such activities may be identified through a variety of approaches, one of them being screening of natural products. Over the last decade, scientific community actively involved in antiviral research have also turned towards the traditional system of folk medicine and Ayurveda, invariably a "cocktail" of natural products, to uncover the scientific basis of their remedial effects.

HIV protease was first suggested as a potential target for AIDS therapy by Kramer (1986), later it was shown that a frame-shift mutation in the protease region of the *pol*-gene prevented cleavage of the *Gag* polyprotein precursor, which is essential for the maturation of the HIV particles. Blockage of HIV protease leads to the formation of immature non-infectious virions (Kohl, 1988).

Based on the presence of the characteristic signature amino acid sequence, Asp-Thr-Gly, was suggested by Toh et al. in 1985 that the protease of HIV might belong to the family of aspartic proteases. This was confirmed through pepstatine A inhibition, an aspartic protease selective inhibitor, (Aoyagi, 1978) and by site-directed mutagenesis of the active site *Asp 25*, which led to abolition of the catalytic activity (Seelmeier, 1988). The aspartic proteases are well characterized group of enzymes that can be found in vertebrates, plants, in addition to in fungi. Examples of proteases from the

aspartic protease class are pepsin, cathepsin D, renin, chymosin, penicillopepsin, and Rhizopus pepsin, which are two-domain enzymes with more that 300 residues in length and contain the Asp-Thr-Gly sequence in each domain which forms the active site, and effectuates the cleavage reaction (Davies, D. R 1990; Pearl, L. H..1987). The HIV protease sequence is no more than 99 amino acid long and contains only one of the required triad Asp-Thr-Gly, which was later confirmed that the active form of the HIV protease is a homo-dimer of 198 amino acids by X-ray crystallographic determinations (Pearl, 1987; Navia, 1989; Wlodawer, 1989; Lapatto, 1989).

The HIV aspartic protease (HIV-PR) 1 is a key enzyme in the virus life cycle and it becomes an indispensable part of conventional HAART or combination therapy for AIDS patients (Balzarini, 1996). This enzyme was earlier perceived as a promising therapeutic target and its inhibition has been successfully used in the treatment of AIDS. Despite this achievement, the emergence of strains resistant to the currently available commercial inhibitors, the high cost of these drugs and its associated toxicity has driven a continuous interest in the development of new inhibitors. The search for new inhibitors often includes high throughput screening programs for detecting HIV-PR inhibitor candidates. In order to identify and evaluate potential inhibitors either from natural sources or from rational designs, some groups have developed HIV-PR activity assays

Numerous data are available which show a great potential of natural products in having natural HIV-Protease inhibitors as, Limonin and Nomilin isolated from *Citrus sps* (Battinelli, 2003), Maslinic acid from *Geum japonicum* (Xu, 1996), Oxygeneted triterpenes (Ganoderic acid-α) from *Ganoderma lucidum* (Mekkawy, 1998), Ursolic acid and Uvaol from *Crataegus pinatifida,*(Min, 1999) and many other compounds are in pipe line.

As a part of our prescreening program to investigate HIV-Protease inhibitory activity of crude plant extracts, various extracts of *Adhatoda vasica* were examined, which is used in Ayurvedic medicinal system to cure diseases known to be of viral origin. An indirect approach was used for screening HIV-Protease inhibitors through pepsin activity inhibition assay from crude plant extract, as many paper support the evidence in the resemblance in the activity of HIV-Protease and Enzyme Pepsin (Toh, 1985; Pearl, 1987; Seelmeier, 1988; Navia, 1989; Wlodawer, 1989; Lapatto, 1989; Davies, 1990; Fitzgerald, 1990).

2. Materials and Methods

Chemical required: Enzyme Pepsin (Himedia), Hemoglobin (Himedia), plants extract, Sodium Acetate tri hydrates (Qualigens), Sodium Chloride (Himedia), Tri Chloro Acetic acid (TCA) (Sdfine), Ethanol, Methanol, Chloroform and experimental plant material. Acetate buffer was prepared by 50 Mm Na Acetate tri hydrate and 0.1 M NaCl with pH- 3.5.

Instruments required

Spectrophotometer - Karry 100, Sigma table centrifuge for 14000 rpm, Rami for 8000 rpm, Micropipette 5-40 µl, 40-200 µl, 200-1000 µl, Eppendorff (1.75ml), Mixer grinder, Mortar pestle, electronic balance.

Plants extract preparation

The plant material was collected from north-west part of Uttar Pradesh state of India. Whole aerial part of fresh plant material was taken, and washed properly with distilled water (DW). 5 g of sample was cut into small pieces and ground properly in mortar and pestle. 10 ml of DW was added to get fine paste and centrifuged for 30 min at 8000 rpm. Supernatant was collected and used as a crude extract of plant material.

Enzyme Pepsin activity inhibition Assay: - Pepsin has a quite close resemblance in proteolytic activity with HIV-1 protease one of key enzyme of HIV-1 life cycle as both of them belong to same Aspartate enzyme family (Maria del *et. al.*, 2004). This enzyme was used as a substitute of HIV-1 protease to check out anti HIV activity of plants extract in this experiment.

Procedure

For this assay, 50µg pepsin, 800µg hemoglobin and different crude plant extracts were taken in 500µl of reaction mixture. The mixture was allowed to incubate at 37^0C, after 20 min 700µl of 5% TCA was added to stop the reaction. It was then centrifuged at 14000 g for 5 min and the supernatant was collected. Optical Density (OD) was recorded spectrophotometrically at 280 nm.

Separate blanks were used for both positive and negative control as well as for sample. For positive control enzyme and substrate were taken and followed the above procedure, and for negative control pepstatin A was taken as a well known inhibitor of both Pepsin and HIV-Protease (Aoyagi, T.1978). Each sample was taken in triplicate, so this assay gives reproducible results.

Several experiments were performed to get optimum activity of enzyme:_

Parameter	optimum range
pH	2- 4
Incubation temperature	37 ^{0} C
Incubation period	20min.
Centrifugation	14000 g.
Reaction volume	500 µL

3. Results

Four extracts (aqueous, ethanol, methanol, and chloroform) of plant were prepared in the current study. Active component was present in aqueous extract while the organic extract have very rare active component with no or negligible toxic effect. Aqueous extract of *A. vasica* extract showed a significant effect on the enzymatic activity of pepsin, that was upto 99% (table-1) and other plant extracts did not show any effect on the enzymatic activity of pepsin. Chloroform extract shown least effect on the enzymatic activity of pepsin.

Table1

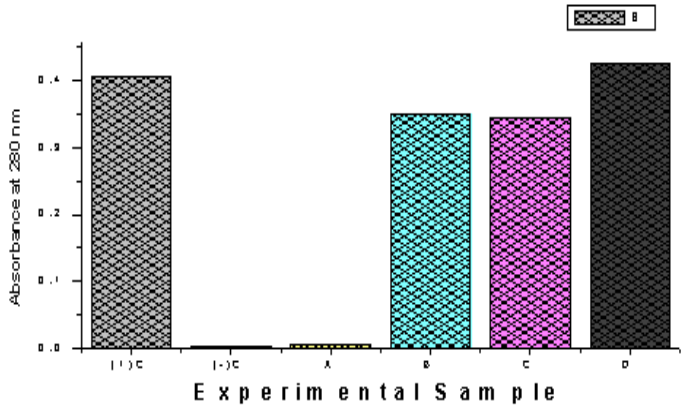
S.No.	Experimental sample	Absorbance at 280 nm
1	(+) Control (absence of any inhibitor)	0.4054 ±0.0085
2	(-) Control (presence of Pepstatin A)	0.0018 ±0.0085
3	Presence of *A. vasica* aqueous extract.	0.0053 ±0.0085
4	Presence of *A. vasica* ethanol extract	0.3506 ±0.0085
5	Presence of *A. vasica* methanol extract	0.3433 ±0.0085
6	Presence of *A. vasica* chloroform extract	0.4253 ±0.0085

4. Discussion and conclusion

AIDS remain the greatest public health challenges in the current world health sector. Currently, no reliable and user friendly treatment can be claimed to combat this diseases. The current anti-HIV drugs that include reverse transcriptase (RT) and protease inhibitors have experienced drug resistance with HIV strains. This imposes the demand for the development of new drugs particularly of plant origin owing to their success as sources of antiHIV drugs. The use of plants for managing different diseases has

become a common practice since time immemorial with most of the people in the developing world relying on traditional medicines for their primary health care including management of HIV/AIDS.

Figure-1 Bar (+) C (positive control) shows pepsin enzymatic activity in the absence of any inhibitor, bar (-) C (negative control) shows approximately 100% inhibition of enzyme activity in the presence of natural inhibitor *Pepstatin A* of enzyme Pepsin, Bar A shows the inhibition of enzyme activity by aqueous extract of *Adhatoda vasica* and bar B, C and D shows inhibitory effect of ethanol, methanol, and chloroform extracts on enzymatic activity of Pepsin respectively



The main purpose of present study is prescreening of HIV-protease inhibitors from *Adhatoda vasica*, which is traditionally used in Ayurvedic system of medicine to cure different kinds of diseases, this eliminates the chances of toxic effect to human system. As shown in figure-1 aqueous extract of *Adhatoda vasica* showed a very significant inhibition on the enzymatic activity of pepsin. Medicinal properties *A.vasica* are already well documented as for cold, cough, whooping cough, chronic bronchitis and asthma. As various previous studies suggested the structural and a functional similarity between pepsin and HIV-Protease, plant extracts which have inhibitory activity of pepsin enzyme should also inhibit the activity of HIV-Protease. HIV-Protease plays a significant part in the replication cycle of HIV, and its dysfunction leads to release of immature and noninfectious virion particles. This makes it an indispensable part of current conventional HAART or combination therapy. This study can append one more option as an alternative inhibitor of HIV-protease to resolve resistance problem of this virus upto some extent. But it still needs further consideration of isolation and chemical analysis of active component of its plant extract.

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